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IN BILIARY OBSTRUCTION
A clinical and experimental study

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by

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The present thesis is based on the following papers:

- I. Separation of lipoprotein X and β -lipoprotein by zonal ultracentrifugation or hydroxyapatite chromatography. Clin. Chim. Acta. 47, (1973), 365-369. In collaboration with B. Danielsson and B.G. Johansson.
- II. Immunochemical studies of lipoproteins in cholestasis. Scand. J. Immunol. 4 Suppl. 2, (1975), 13-18. In collaboration with R. Ekman and B.G. Johansson.
- III. An abnormal high density lipoprotein in cholestatic plasma isolated by zonal ultracentrifugation. FEBS Letters 2, (1975), 180-184. In collaboration with B. Danielsson and R. Ekman.
- IV. Characterization of high density lipoproteins in human cholestasis. Scand. J. Clin. Lab. Invest. (Submitted for publication). In collaboration with B. Arnesjö, B. Danielsson and B.G. Johansson.
- V. Zonal ultracentrifugation of plasma lipoproteins from normal and cholestatic pigs. Clin. Chim. Acta. 65, (1975), 187-196. In collaboration with B. Danielsson, R. Ekman and B.G. Johansson.
- VI. Abnormal low density plasma lipoprotein occurring in dogs with obstructive jaundice. FEBS Letters 63, (1976), 33-36. In collaboration with B. Danielsson, R. Ekman and B.G. Johansson.
- VII. Plasma lipoprotein changes in experimental cholestasis in the dog. Clin. Chim. Acta. (Submitted for publication). In collaboration with B. Danielsson, R. Ekman and B.G. Johansson.

The above articles are referred to in the text by the Roman numerals.



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Abbreviations

Apo A I, A II	Apoproteins A I, A II
Apo B	Apoprotein B
Apo C I, C II, C III	Apoproteins C I, C II, C III
Apo D	Apoprotein D
Apo E	Apoprotein E (arginine-rich peptide)
CLP	Cholestatic lipoprotein in dogs
HDL	High density lipoproteins
HDL ₁ -C	Cholestatic high density lipoproteins
IDL	Intermediate density lipoproteins
LP A	Lipoprotein A (lipoprotein family containing only apo A I and A II)
LP B	Lipoprotein B (lipoprotein family containing only apo B)
LP C	Lipoprotein C (lipoprotein family containing only apo C I, C II and C III)
LP-X	Lipoprotein X
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoproteins
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulphate
VLDL	Very low density lipoproteins

BACKGROUND

Plasma lipoproteins

Lipoproteins in plasma transport triglycerides, esterified and unesterified cholesterol and phospholipids through the extracellular space. The lipoprotein particles, which vary widely in size and composition, are composed of lipids and a protein moiety containing various apolipoproteins. Together with phospholipids and unesterified cholesterol various combinations of apolipoproteins (apo A, apo B, apo C, apo D and apo E) (37, 47, 79, 86) constitute the hydrophilic outer layer, and triglycerides and esterified cholesterol make up the bulk of hydrophobic core. The term apo A comprises two structurally different proteins (A I and A II) and the designation apo C, three (C I, C II, C III). This extension of the scope of the terms apo A and apo C which was introduced by Alaupovic et al (2), is now generally accepted. All apolipoproteins are structurally different, as reflected in differences in their immunochemical specificity.

Lipoproteins are usually separated according to their physicochemical properties, such as density (ultracentrifugation) or electrophoretic rate of migration. Recently, gel filtration in agarose or dextran gels has been introduced for the separation of lipoproteins differing in particle size. Lipoproteins can be separated also immunochemically by methods utilizing their differences in apolipoprotein composition. Reviews of plasma lipoproteins have been published by several authors (45, 53, 55).

Flotation analysis in analytical or preparative ultracentrifugation (cf. 46) can separate lipoproteins into various density classes called very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and very high density lipoproteins (VHDL) (Table I). These classes can in turn be divided into subclasses. Thus, for example HDL can be divided into HDL₂ and HDL₃.

The most common method for separating lipoproteins in preparative ultracentrifugation is so-called differential flotation. This is a laborious procedure using predetermined density limits which do not a priori agree with the true density distribution. The chylomicrons are separated by centrifugation of plasma at 1.006 kg/l 25 000 - 50 000 x g for 30 minutes. By prolongation of such centrifugation at least 20 hours the whole VLDL class is obtained. In order to obtain LDL and HDL the density of the plasma sample is then stepwise adjusted to 1.063 and 1.21 kg/l by addition of sodium bromide. After each adjustment the sample is centrifuged for at least 24 hours at 100 000 g. The material floated is separated, and the infranatant adjusted to the next density step and centrifuged. The various fractions are often recentrifuged to obtain a higher purity. Complete separation of the various lipoprotein classes requires more than one week.

Table I

Classification of plasma lipoproteins. (Data mainly from Hamilton-Kayden Ref. 29 and Levy Ref. 45)

Lipoprotein class	Density (kg/l)	Size (Å)	Electrophoretic mobility (agarose gel)	Main apoproteins
Chylomicrons	< 0.95	800-10 000	Origin	B, C
VLDL	0.95-1.006	300-800	pre- β	B, C, (E)
IDL	1.006-1.019	200-300	pre- β - β	B, (C, E)
LDL	1.019-1.063	180-220	β	B
HDL ₂	1.063-1.125	85-100	α	A, (C)
HDL ₃	1.125-1.210	70-90	α	A, (C)
VHDL	> 1.21		α , pre- α ?	A

Owing to the predetermined density limits differential centrifugation separates lipoproteins into different density classes and does not depict the continuity of the lipoprotein spectrum; in certain cases it may even leave a false impression of the density distribution. This disadvantage can be avoided by centrifugation in a continuous density gradient. If the samples are small, they may be centrifuged in swing-out rotors, but if they exceed 1 ml, a specially designed zonal rotor must be used (3). Zonal centrifugation, which has been extensively used in the present work can thus separate all classes of lipoproteins within 24 hours and with a continuous recording of the density distribution (38, 90).

Lipoproteins can also be separated according to their electrophoretic behaviour. Thus, electrophoretic separation of lipoproteins in agarose gel results in fractions called β -lipoprotein, pre- β lipoprotein and α -lipoprotein (33, 57). These fractions normally correspond roughly to LDL, VLDL and HDL. Chylomicrons remain in the application slit.

Also the apoprotein content of lipoproteins has been suggested as a classification principle by Alaupovic et al (2). According to these authors, there are lipoproteins (so-called lipoprotein families) that are dominated by apo A (LP A), apo B (LP B), apo C (LP C) and apo D (LP D). The term lipoprotein family is not yet generally established, but has been used to some extent in this work in the characterization of LDL and HDL. The relation between the various classification principles and the particle size and content are given in Table I.

The structural similarities of different lipoprotein classes is assumed to reflect their metabolic relationships (29). From a functional point of view, the lipoproteins can be divided into two (possibly three) systems: the VLDL-IDL-LDL system, of which the major function is to transport endogenous triglyceride from the liver to peripheral tissues; the

chylomicron - chylomicron remnant - LDL system, which transports exogenous (dietary) triglycerides to peripheral tissues, and which resembles the VLDL-IDL-LDL system; and the HDL system which is assumed to have a role in the regulation of the processes involved in the metabolism of VLDL and chylomicrons. The HDL system has also been suggested to transport cholesterol from peripheral tissues to the liver (23).

Triglyceride-rich lipoproteins are secreted by the liver (VLDL) and intestine (chylomicrons) into the plasma compartment. The metabolism of these lipoproteins, involves the passive transfer of apoproteins, mainly apo C and apo E from the HDL system, as well as hydrolysis and elimination of part of the triglyceride component (29). The lipolytic step occurs at the endothelial lining of the capillaries in most peripheral organs and is catalyzed by the enzyme, lipoprotein lipase (36). These processes result in the transformation of VLDL to a short-lived lipoprotein of intermediate density (IDL) and of chylomicron to a chylomicron remnant. Whether IDL and chylomicron remnants are identical is under debate (64). The further metabolism of these lipoproteins has not been elucidated to the same extent, but involves further modification of the protein component as well as of the lipid constituents. There is evidence that the catabolism of the IDL/chylomicron remnant occurs in the liver (64), and it has been suggested that the so-called liver lipase plays a role in this step (17). These processes lead to the formation of LDL particles, which are eliminated from plasma and catabolized presumably in extrahepatic tissues (81).

The metabolism and functions of the HDL system are less well-known. It is agreed that HDL particles are synthesized and secreted by the liver. The native HDL particles are transformed by their participation in the above-mentioned transport of apoproteins and by the action of the lecithin cholesterol acyltransferase (LCAT) activity, which is associated with the HDL particles. The half-life of HDL has been reported to be 3 - 4 days; their fate is unknown (29, 71).

Disturbances of the metabolism of lipoproteins are common. Most of such disturbances result in hyperlipoproteinemia and only rarely, in hypolipoproteinemia (34). A few cases of primary hypolipoproteinemia, of α - β -lipoproteinemia and α -lipoprotein deficiency or Tangier's disease are on record (34).

Five types of hyperlipoproteinemias are recognized by Fredrickson (20). These types are said to occur in primary familial diseases, but occasionally also secondarily to other conditions such as diabetes, alcoholism, pancreatitis and nephrosis. The most common hyperlipoproteinemias are type II, in which B lipoprotein (LDL) is increased, and type IV in which pre- β -lipoprotein (VLDL) is increased. In type I the chylomicrons are increased, while in type V the chylomicrons as well as pre- β -lipoprotein are increased. On the other hand, the

so-called type III hyperlipoproteinemia is considered to be a dyslipoproteinemia with an apparently qualitatively abnormal electrophoretic pattern. In this condition there are lipoprotein particles with a density between that of VLDL and of LDL (61). Another dyslipoproteinemia with abnormal lipoproteins is seen in a cholestasis condition in which an abnormal lipoprotein (LP-X) occurs.

So far, hyperlipoproteinemias have been most extensively studied in the VLDL, LDL range because of the high correlation between atherosclerosis and increased correlations of especially IDL and LDL, and the assumption that the atherogenetic process is indeed due to the increased lipoprotein concentrations. Also hyperlipoproteinemia of HDL type has been described (25), but its significance is obscure.

Lipoproteins in obstructive jaundice

It has long been known that conspicuous changes in plasma lipid concentrations are common in liver disorders. More than a century ago Flint (18) observed that the blood cholesterol concentration was increased in obstructive jaundice. Subsequent studies correlating plasma lipids with various liver diseases revealed dramatic changes in plasma lipids and lipoproteins in patients with intra- or extra-hepatic biliary obstruction (10, 16, 49). At that time knowledge about the way lipids were carried in plasma was scanty.

Kunkel et al (42) studied plasma lipid and β -globulin concentrations for any correlation and were among the first to suggest that the hyperlipoproteinemia and increased phospholipid concentrations in biliary obstruction were related. Gofman et al (26) described the marked increase in the serum LDL as a characteristic of the ultracentrifugal lipoprotein pattern in obstructive jaundice. Several investigators demonstrated that the increased concentration of LDL was sometimes accompanied by a decreased concentration of HDL (59, 80). The increased serum concentrations of the low density lipoproteins in obstructive jaundice was shown by Eder et al (13) to be due to the presence of an abnormal lipoprotein that could be isolated from the Cohn fraction IV + V + VI while normal LDL is recovered in fraction I + III. Switzer (84) fractionated the low density lipoprotein fraction of patients with primary biliary cirrhosis with the use of antibodies to normal low-density lipoprotein and demonstrated the presence of normal LDL as well as of an abnormal lipoprotein called "obstructive lipoprotein". It was rich in phospholipid and unesterified cholesterol. Triglycerides were virtually absent and there was only a little protein. Antiserum against it reacted with very low density lipoprotein, but not with normal low density or with high density lipoprotein.

Switzer's results were confirmed and extended by Seidel et al (73, 74). They gave the abnormal lipoprotein the name LP-X, believing initially that it contained a protein moiety of unique composition consisting

of albumin and a specific apoprotein, apo X with an extraordinary lipid binding capacity. After delipidisation, however, both immuno-precipitation and polyacrylamide gel electrophoresis suggested that apo X consisted of apo C proteins, mainly C I.

Lipoprotein X differs in many respects from that of the normal lipoproteins. It has a unique lipid composition characterized by high content of phospholipids and unesterified cholesterol but contains only traces of triglycerides. The protein moiety consists of albumin and apoprotein C (73, 74).

In electron microscopy LP-X appears as discs with a flattened appearance and a great tendency to form rouleaux (28). The thickness of the discs is about 100 Å and their diameter 400 - 600 Å. On electrophoresis in agar LP-X migrates towards the cathode whereas the other lipoproteins move in the anodal direction (73, 74).

High density lipoproteins also undergo changes in biliary obstruction (7, 77, 80). It is known that the plasma HDL decreases in cholestasis but the various HDL species have not been investigated as thoroughly as LP-X. HDL gradually decreases in biliary obstruction and if the obstruction is of long duration, the density distribution is usually profoundly changed. Alterations within the HDL₃ class in biliary obstruction have been investigated immunoelectrophoretically by Seidel et al (77) who have shown heterogeneity in HDL₃ and by Quarfordt et al (62) who also found abnormal cholestatic HDL.

The mechanism of the above lipoprotein changes has been investigated in animals with induced obstructive jaundice. An analogous component with a lipid composition similar to that of human LP-X has recently been observed in plasma from dogs with experimental biliary obstruction (54, 63, 65). Also ligation of the common duct in rats is followed by changes in the LDL-region resembling those of human cholestasis (9, 75).

Research in the literature failed to reveal any report of HDL changes in obstructive jaundice in pigs or dogs.

The aim of the present work was to isolate and characterize cholestatic lipoproteins from various lipoprotein density classes. For this purpose the convenient zonal ultracentrifugation technique was mainly employed. Characterization of lipoproteins were performed by studies of the lipid and apoprotein composition of the lipoprotein fractions obtained.

In order to study the formation and fate of abnormal lipoproteins occurring in bile obstruction it was found necessary to use experimentally induced cholestasis in animals as a possible model for the lipoprotein changes in human cholestasis. Consequently investigations of lipoprotein changes in dogs and pigs were performed. The changes in these animals were to some extent similar but by no means identical to those found in patients with obstructive jaundice.

MATERIALS

Clinical material

In I, II, III and IV and the material presented in this paper, blood samples were obtained from patients with extrahepatic biliary obstruction and from healthy volunteers after 12 - 14 hours fasting. The diagnosis in the patients with obstructive jaundice was confirmed by conventional laboratory tests, liver biopsy and surgical procedures.

Animals

Pigs weighing 20 - 25 kg were fed on a diet rich in carbohydrate and fat ad libitum for 14 days, before ligation of the common bile duct (V).

In VI and VII one to two-year-old mongrels of either sex and weighing between 18 and 22 kg were used. The dogs were fed on commercial dog food before and after ligation of the common bile duct.

Whole blood was collected from animals that had been fasting for 12 hours. The samples were collected from a catheter permanently inserted in the vena jugularis and emptying into a vessel containing Na₂EDTA as anticoagulant.

Preparation of lipoproteins for antiserum production

Human, dog and pig LDL and HDL were prepared by differential flotation, as described by Havel et al (31). Human LP-X was prepared by a combination of polyanion precipitation (73, 74) and immunoabsorption of contaminating lipoprotein B (84) according to the following scheme:

1. precipitation of the low density lipoproteins with heparin and MnCl₂;
2. immunoprecipitation of apo B containing lipoproteins with an immunoglobulin preparation of rabbit anti-human B lipoprotein; and
3. flotation of LP-X for 24 hours at a density of 1.063 kg/l

The purity of the lipoprotein preparations was checked with agarose gel electrophoresis stained with Sudan Black B and Coomassie Brilliant Blue (33).

Apoproteins A I and A II were isolated from delipidised HDL by gel filtration, as described by Scanu et al (15, 72).

Apoprotein C I was prepared from delipidised VLDL by ion exchange chromatography essentially as described by Shore and Shore (79). The purity of these apoproteins was controlled with urea polyacrylamide gel electrophoresis (12). The identity of apoproteins A I, A II and C I was also confirmed by Prof. A. Scanu, Division of the Biological Sciences and the Pritzker School of Medicine, University of Chicago.

Production of antisera

Antisera were raised in rabbits by intramuscular injection of 0.2 - 0.5 mg of purified lipoproteins emulsified with one volume of complete Freud's adjuvant. The injections were repeated after 3 and 6 weeks and thereafter every 6th week. Antibody responses were generally obtained 2 weeks after the second injection. The antisera were absorbed as described below in order to render them monospecific. The amounts of antigens to be added for complete absorption of contaminating antibodies were carefully determined by pilot absorption experiments.

After immunization with human HDL anti-HDL (anti- α -lipoprotein) was obtained by absorption with the infranatant fraction obtained by ultracentrifugation of normal serum at a density of 1.25 kg/l for 72 hours and with pure normal LDL. The antiserum contained antibodies to A I and A II, but showed no reaction with LDL or VLDL. Anti-LDL (anti- β -lipoprotein) was rendered monospecific for B protein by absorption with the infranatant fraction of normal serum centrifuged at 1.063 kg/l used for preparation of HDL. Anti-LP-X was also absorbed with this fraction as well as with normal LDL. Anti-LP-X contained antibodies to C proteins but showed no reaction with B protein. Anti-A I was absorbed with A II and with the infranatant fraction of normal serum centrifuged at 1.21 kg/l. Anti-A II was absorbed with A I. Finally anti C I was absorbed with the infranatant fraction of normal serum centrifuged at 1.21 kg/l. The immunoglobulin fraction was prepared from all antisera with the octanoic acid precipitation technique described by Steinbuch and Audran (83).

Antisera against porcine and canine LDL and HDL were raised by immunization of rabbits with lipoprotein fractions purified by preparative ultracentrifugation in essentially the same way as that described for human lipoproteins. The antisera against HDL were absorbed with the infranatant fraction obtained by ultracentrifugation at 1.21 kg/l and with purified LDL. The anti-LDL serum was absorbed with the infranatant obtained at a density of 1.07 kg/l. On crossed immunoelectrophoresis both anti-HDL and anti-LDL were found to contain single immunoprecipitates when tested against porcine or canine plasma, respectively. The antisera against human HDL and LDL cross-reacted distinctly with the corresponding canine lipoproteins.

METHODS

Laboratory studies. Determination of the serum bilirubin (S-Bil), alkaline phosphatases (S-ALP), γ -glutamyltransferase (S-GT), alanine-amino-transferase (S-ALAT) and aspartat-amino-transferase (S-ASAT) activities in patients and animals with conventional methods were made at the routine clinical chemical laboratory, University Hospital, Lund. Protein concentrations were determined with a modification of the Lowry method (41).

Lipoprotein analysis. Lipoprotein electrophoresis was done as described by Johansson (33). α - and β -lipoproteins were estimated by electro-immunoassay, as described by Laurell (43). Crossed immunoelectrophoresis was carried out according to Ganrot (21). Crossed immunoelectrophoresis with an intermediate antibody-containing gel was performed as described by Axelsen (4). Agar gel electrophoresis in Difco Bacto Agar with subsequent polyanion precipitation as described by Seidel et al (78) was used for detecting LP-X.

Zonal ultracentrifugation was performed in a Beckman model L2-65B ultracentrifuge equipped with a Ti-14 zonal rotor. The Ti-14 rotor has a volume of 650 ml and is capable of 172 000 g at 48 000 rpm. Density gradients were made by mixing 0.01 M Tris buffer Ph 7.4 containing 1 mM EDTA with sodium bromide dissolved in the same buffer ($d = 1.200$ or 1.300 kg/l). The solutions were filtered and degassed before use. Density gradients linear with respect to rotor volume were generally employed although any gradient shape could be produced with the gradient machine used. The gradients were made by mixing buffer and sodium bromide solution by two peristaltic pumps programmed by a curve-follower. The centrifugation technique was essentially that described by Wilcox et al (90).

VLDL and LDL were isolated in a 600 ml gradient with a density range of $1.000 - 1.150$ kg/l. The plasma samples (usually 15 - 30 ml) were adjusted to a density of 1.160 kg/l with solid sodium bromide before introduction and followed by a cushion of sodium bromide solution ($d = 1.170$) to fill the rotor completely. The centrifugation was performed at 48 000 rpm (max RCF 172 000 g) at $+15^{\circ}\text{C}$. The centrifugation time was 1.5 h for human plasma samples. Since the high hydrated densities of canine and porcine LDL are higher than those of human LDL, longer centrifugation time (3 h) is required for optimal separation of VLDL-LDL.

HDL was isolated in a 600 ml gradient with a density range of $1.000 - 1.290$ kg/l. The density of the sample was adjusted to 1.295 kg/l with solid sodium bromide and sodium bromide solution ($d = 1.300$ kg/l) was used as cushion. The centrifugation was carried out at 46 000 rpm

(max RCF 158 000 g) at +15°C for 15 to 20 hours. Human HDL was also isolated in a more complex gradient (II and IV).

The rotor was center-unloaded at 3 000 rpm and the rotor content was continuously monitored for UV-absorbance at 280 nm with a Uvicord II (LKB-Products, Stockholm, Sweden) via a transmission absorbance converter. 20 ml fractions were collected and the densities of the fractions were checked by weighing 10 ml aliquots.

Gel filtration on Sephadex G-200 superfine, particle size $20 \pm 2 \mu\text{m}$, obtained by dry elutriation (14) was done at +4°C in 0.05 M Tris-HCl pH 7.4, containing 0.01 M EDTA and 0.2 M NaCl. Columns, 5 x 90 cm, (Pharmacia, Uppsala, Sweden) designed for upward flow technique were used. The flow rate was kept at 12 - 15 ml/h. The effluent was collected in fractions and the absorbance of each fraction was measured at 280 nm.

Hydroxyapatite chromatography was carried out with Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Calif.) which was suspended in 0.05 M sodium phosphate buffer pH 7.4 and packed in columns 2.5 cm in diameter. The lipoprotein components were eluted with a linear gradient of sodium phosphate buffer pH 7.4, using two communicating chambers containing (I) 200 ml 0.05 M phosphate buffer and (II) 200 ml 0.65 M phosphate buffer. Elution was performed at a flow rate of 15 ml per hour at +4°C. The effluent was collected in 5 ml fractions after recording the absorbance at 280 nm with a Uvicord II.

Apoprotein composition. Apolipoproteins were separated by SDS-gel electrophoresis in a continuous buffer system (88) (III) or in a discontinuous system (22,35) (IV, V, VI, VII). The samples were usually reduced with 2-mercaptoethanol and heated at 90°C for three minutes. A number of well-characterized proteins were used as molecular weight markers. Electrophoresis of the apoproteins was also performed according to Davies (12) in 7.5% polyacrylamide gels containing 8 M urea after delipidization with tetramethylurea. After electrophoresis the proteins were stained with Coomassie Brilliant Blue R 250. Densitometric recordings were made with Zeiss PMQ II spectrophotometer equipped with a gel scanner (type ZK 4).

Lipids were extracted from the lipoprotein fractions with chloroform: methanol 1/1 (v/v) (19). Phospholipids were determined on an aliquot of the chloroform extract by the colorimetric method described by Itaya and Ui (32), as modified by Belfrage et al (6) or by the colorimetric method described by Bartlett (5) (IV).

The lipid classes were separated by thin layer chromatography on silica gel using petroleum ether (b.p. 30-60°C) - ethyl ether - glacial acetic acid (90/10/1, v/v/v) as the mobile phase. The tri-glyceride cholesterol ester and cholesterol fractions were eluted from the silica gel with chloroform: methanol 8:1 (v/v). Cholesterol

was determined by the colorimetric method described by Abell et al (1), and triglycerides with the enzymatic fluorometric methods of Laurell and Tibbling (44) or the enzymatic-colorimetric method of Röschlau et al (70) (IV).

RESULTS

Isolation of lipoproteins of VLDL-LDL classes

Lipoproteins from cholestatic plasma were fractionated by the polyanion precipitation technique of Burstein et al (8) for separation of VLDL and LDL from normal plasma. Zonal ultracentrifugation of this fraction revealed a pattern with three distinctly separated fractions designated I, II and III in paper I. Immunochemical analysis showed that the densest of these fractions (No III) reacted with anti- β -lipoprotein in electroimmunoassay, whereas no precipitates were found in the other main fraction (No II). This latter fraction, which was not found on analysis of normal plasma, was assumed to be identical with LP-X on account of its lack of B protein and its reaction with antibodies to LP-X. Later studies of the lipid composition showed the characteristic pattern of lipoprotein X (unpublished results). The apoprotein pattern was characterized by a predominance of C proteins; apoprotein E and albumin could also be detected. The albumin content was determined by electroimmunoassay and was found to be around 10 per cent of the protein part (Ekman et al, unpublished results) which is considerably less than that reported by Seidel (74).

The lightest fraction (No I) obtained by zonal ultracentrifugation of cholestatic plasma contained no material reacting with antisera to LP-X or to lipoprotein B. Also the amounts of lipids were small. It is possible that this fraction contained very small amounts of aggregated lipid or lipoprotein material with a heavy light-scattering effect, producing the impression of substantial amounts of material. Analogous fractions were also obtained when normal LDL was centrifuged after polyanion precipitation, and it should therefore be concluded that the phenomenon was not characteristic of lipoproteins of cholestatic plasma.

In later studies cholestatic plasma was ultracentrifuged without preceding polyanion precipitation of VLDL and LDL. It revealed not only the pattern analogous to that described in paper I, and occurring in 12 out of 25 plasma samples (Fig. 1 A) but also several other patterns. In 5 patients two partially separated fractions were obtained besides the normal B-containing LDL, which was more or less contaminated with abnormal LP-X containing components (Fig. 1 B). A third pattern obtained in 4 patients showed a continuous distribution of material that reacted with anti LP-X without noteworthy enrichment in any particular density interval. The remaining 4 patients exhibited individual profiles that would not fit into any of the patterns described above.

In a study on LDL from patients with severe obstructive jaundice Patsch et al (60) using zonal ultracentrifugation, demonstrated two abnormal low density lipoproteins in the LDL interval called LP-X₁ and

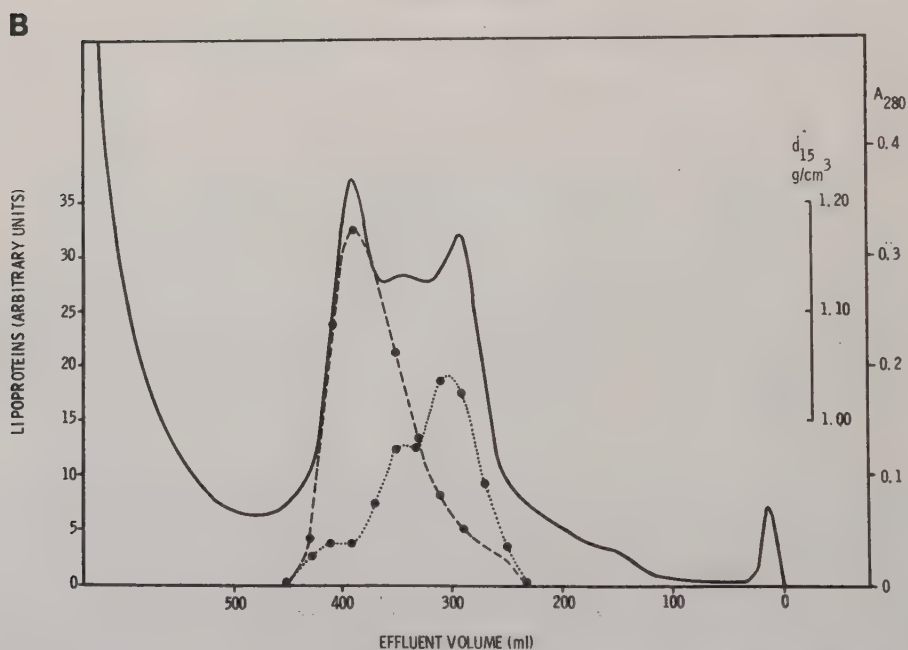
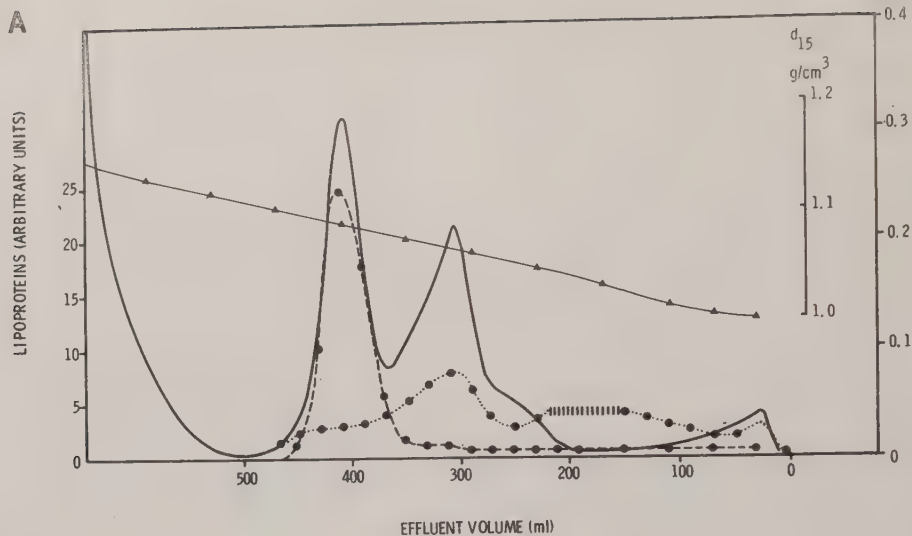


Fig. 1. Zonal ultracentrifugation in a continuous sodium bromide gradient linear with respect to volume (1.00-1.15 g/cm³) A) 15 ml and B) 18 ml plasma from two patients with cholestasis. The rotor (Ti-14) was run at 1.5 h at 48 000 r p m at 15°C. — A_{280} , ▲ — density at 15°C, ● — — — ● and ● ····· ····· ● electroimmunoassay against anti-LDL and anti-C respectively.

LP-X₂. Both had an apparent hydrated density lower than that of normal LDL, which is consistent with the results we obtained with plasma samples from patients with obstructive jaundice. To isolate these lipoprotein fractions Patsch et al had to use zonal ultracentrifugation combined with Cohn fractionation. They found a significantly lower protein content and a higher TG proportion in LP-X₁ and LP-X₂. Nothing is known about the significance of this additional heterogeneity of lipoproteins lighter than normal LDL, although it seems to occur only in severe obstructive jaundice.

An increased heterogeneity compared to earlier results has been demonstrated by Kostner et al (40) who used hydroxyapatite-chromatography for further fractionation of LDL isolated by differential flotation after precipitation with polyanion. They recovered the following fractions: LP-X, a triglyceride-rich LP Y and an abnormal LP B. The first fraction had the properties of LP-X as described by Seidel (74). The ultracentrifugal properties of these three components indicated a heterogeneity similar to that discussed above. Closer inspection of data concerning lipid and protein compositions (40), however, clearly showed considerable discrepancies between fractions with similar behaviour on flotation but isolated by different methods. It is not clear whether these discrepancies are ascribable to differences in the clinical materials studied (e.g. duration and intensity of the obstructive jaundice) or to differences between the isolation procedures used.

Although zonal ultracentrifugation substantially improved resolution of lipoproteins of various densities, the density distribution of B-containing lipoprotein and abnormal components in cholestasis did not permit the isolation of LP-X in more than half of patients studied, and even then it was sometimes contaminated with B protein. For the separation of abnormal components and lipoprotein B zonal ultracentrifugation must be combined with other methods in such cases. As demonstrated in paper I, hydroxyapatite chromatography seems to be suitable for this purpose. The value of hydroxyapatite chromatography combined with differential centrifugation was recently demonstrated in a paper by Kostner et al (40).

Immunochemical studies of lipoprotein B and lipoprotein X

Changes in the plasma β -lipoprotein-pre- β -lipoprotein patterns in cholestasis were easily demonstrated by crossed immunoelectrophoresis as shown in paper II. The normal plasma pattern revealed two peaks against anti- β -lipoprotein, corresponding to β -lipoprotein and pre- β lipoprotein. The pre- β fraction was often missing in the cholestatic plasma pattern. This might be due to a decrease of VLDL in obstructive jaundice, but it has also been suggested that cholestatic VLDL acquires a β -mobility on account of altered apoprotein composition (77). Results

of zonal centrifugation (cf. Fig. 1) indicated that most cholestatic patients have low levels of VLDL.

The unique apoprotein composition of lipoprotein X with a dominance of apo C and absence of apo B should offer good possibilities for detecting and quantifying LP-X. Crossed immunoelectrophoresis of cholestatic plasma against anti-lipoprotein X, containing abundant antibodies against C proteins, showed in the β -lipoprotein region a narrow peak not visible in normal plasma which was identical to lipoprotein X (II). This method could be used for detection and semi-quantitative determination of LP-X. A drawback with such a method would be the presence of C proteins belonging to the normal VLDL-LDL system, which would presumably interfere with this analysis. Therefore, crossed immunoelectrophoresis with an intermediate gel (4, 39) was used for more specific analysis. Antiserum against LP B was included in the intermediate gel. This antiserum served as a barrier to all lipoproteins containing B e.g. VLDL-LDL, while lipoprotein X not containing B passed through this gel and was precipitated in the upper gel containing anti-lipoprotein X (or any antiserum against C proteins). The intermediate gel technique is also very useful for the determination of LP-X by electroimmunoassay (unpublished observations). But the results must be interpreted with caution, since LP-X varies in composition and physicochemical behaviour.

Other methods have been developed for quantitative determination. Magnani and Alaupovic (48) developed a quantitative method based on the measurement of the phospholipid content of LP-X after electrophoretic separation of LP-X from the normally occurring lipoproteins. Ritland (68) described a method based on the estimation of labelled free cholesterol in the LP-X fraction after equilibration of serum or plasma with labelled cholesterol followed by electrophoretic separation of the lipoproteins. Neubeck and Seidel (56) have recently developed a method of quantifying LP-X based on densitometry of the precipitation areas obtained after serum electrophoresis in agar gel followed by precipitation with polyanions.

However, all these methods have the same disadvantage as the intermediate gel technique, since, as pointed out above, it varies in composition and physicochemical behaviour.

Characterization of high density lipoproteins in human cholestasis

The changes of HDL in cholestasis consistently comprised a decrease of the α -lipoprotein zone on agarose gel electrophoresis. In cases with fairly long (2-3 weeks) obstructive jaundice the α -lipoprotein zone had completely disappeared (II, IV). Electroimmunoassay also showed a decrease of α -lipoprotein which was, however, never as pronounced as the findings on lipoprotein electrophoresis indicated. The immuno-precipitates had changed in morphology, their outlines were fuzzy

and they stained only poorly with lipophilic dyes, such as Sudan Black. These observations suggested that electroimmunoassay was not suitable for accurate immunochemical determinations of α -lipoprotein in cholestasis because of probable qualitative changes in the HDL class. This assumption was verified by crossed immunoelectrophoresis against anti- α -lipoprotein, which showed profound differences from the normal α -lipoprotein pattern. A complicated profile was obtained with 3-4 peaks electrophoretically distributed from the normal α -lipoprotein region to the pre- β -region.

The peak with the most anodic localization might be very similar to that of normal α -lipoprotein. The other components not demonstrable in normal plasma were studied further with immunoelectrophoretic methods. The use of the intermediate gel technique of Axelsen (4) showed that one of the components not invariably present had a distinct immunoreactivity against anti-apo A II, while A I was apparently missing. So far no such component has been isolated from HDL, but its presence in cholestatic HDL was indicated by earlier studies of Seidel et al (77), who used classical immunoelectrophoresis and demonstrated a dissociation of the main HDL apoproteins in cholestasis. The observation is of interest in the light of the lipoprotein family concept (37) which claims a close relationship between A I and A II (LP A) in HDL.

The most cathodal peak seen in crossed immunoelectrophoresis against anti- α -lipoprotein, was also visible on similar analysis using anti-serum against apo C I (II, IV), indicating the presence of considerable amounts of this peptide. This fraction was identified by addition of an abnormal HDL fraction (HDL₁ or HDL₁-C) isolated from cholestatic plasma (III). This isolated fraction had the same electrophoretic mobility as the slow fraction on crossed immunoelectrophoresis, and addition of the fraction to cholestatic plasma resulted in a considerable enlargement of the slow peak.

The changes of α -lipoproteins demonstrated immunochemically were further studied by zonal ultracentrifugation of HDL in cholestatic plasma (IV) which revealed both quantitative and qualitative differences from normal plasma HDL. Instead of the normal pattern of HDL₂ partially separated from the dominant HDL₃ subclass, only small amounts of material were found in this density region with disappearance of the normal distinct demarcation of HDL₂ and HDL₃ (Paper III, IV). In addition, there appeared a small but distinct HDL₁ fraction, called cholestatic HDL₁ (HDL₁-C). It had a density of about 1.10 kg/l and was not demonstrable in normal plasma.

The cholestatic HDL fractions including HDL₁-C were analysed for their apoprotein and lipid composition. HDL₁-C showed the most conspicuous difference from normal HDL. In this fraction the dominating apoprotein had a molecular weight of about 35 000 daltons, as judged from the apoprotein patterns on SDS-polyacrylamide gel electrophoresis. It is

reasonable to assume that this component is the arginine-rich peptide (79) or apo E (86). Increased amounts of apo E have been found by Utermann et al (86) in cholestatic HDL₂, isolated by differential flotation. No apoprotein B was found immunochemically in HDL₁-C, a finding that excluded contamination of LDL. Other apoproteins occurred in rather variable amounts. Generally the amounts of A I and A II were largely equal. In some preparations the amounts of C I appeared to be larger than in normal HDL, which is in accordance with the appearance of HDL₁-C on crossed immunoelectrophoresis against anti-C I. The apoprotein composition was also studied with urea-polyacrylamide gel electrophoresis, and confirmed the results obtained on the SDS-gels.

The lipid composition of HDL₁-C was also quite different from that found in normal HDL. Polar surface lipids (cholesterol and phospholipids) constituted 80% of the total lipids, while the minor remaining part comprised triglycerides and cholesterol esters, which are usually found in the core of lipoproteins (c.f. IV, Table II).

A similar protein and lipid composition was found also in cholestatic HDL₂, while the HDL₃ fraction was more like its normal counterpart.

Gel filtration of cholestatic plasma and subsequent analysis of the distribution of material immunoreactive to anti-HDL also demonstrated pronounced alterations of the particle size distribution. A small component with a K_{av} -value smaller than normal HDL was eluted first. On crossed immunoelectrophoresis this fraction behaved in the same way as HDL₁-C. The other main peak was eluted later than the main part of normal HDL and on crossed immunoelectrophoresis it behaved like normal HDL. The results were in principle comparable to those obtained by Blomhoff (7), who studied cholestatic HDL, isolated by differential ultracentrifugation, on Sephadex G-200. The direct gel filtration of plasma was used in the present study in order to diminish the risk of transformation of the HDL particles by the time-consuming ultracentrifugation step.

Although electron microscopy was not used in our study it is reasonable to assume that HDL₁-C, which obviously corresponds to the large size particle fraction in the gel filtration experiments, is similar to the disc-shaped particles shown to be present in cholestatic HDL (29). This assumption is strengthened by the findings of an abundance of polar surface lipids in HDL₁-C, which is consistent with a discoid shape of the HDL₁-C particles. The dimensions of the particles found by Hamilton and Kayden (29) also agree with the small K_{av} found in our gel filtration experiments.

Plasma lipoprotein changes in experimental cholestasis

In order to elucidate the mechanism of the altered lipoprotein metabolism in human cholestasis, obstructive jaundice was induced in animals

by ligation of the common duct.

Preliminary experiments were performed on rats. Although electrophoretic studies showed that ligation gave rise to lipoprotein changes, observed on lipoprotein electrophoresis, the obstruction seemed to persist only for one week as judged from liver function tests and lipoprotein patterns. This might be due to recanalization of the biliary tract. Therefore, other species were chosen for the experiments. Since the zonal ultracentrifugation procedure requires comparatively large plasma samples, larger animals were preferred. The experiments comprised studies on pigs and dogs.

Cholestasis in pigs

The material consisted of seven pigs of Swedish land race. After ligation of the common bile duct in six of the animals a severe hyperbilirubinemia developed within a few days. Maximal values were seen about one week after operation. Histological examination of the liver at autopsy after 14 days confirmed the presence of cholestasis. No changes in the liver function were found in the sham-operated animal (V).

Despite the obvious signs of cholestasis agar gel electrophoresis according to Seidel et al (78) did not reveal any lipoprotein with the cathodal migration characteristic of human LP-X. Nor were any changes found in the lipoprotein patterns in agarose gel. Electroimmunoassay and crossed immunoelectrophoresis of porcine plasma against anti- α -lipoprotein and anti- β -lipoprotein immune sera revealed no significant changes.

In spite of these negative findings zonal ultracentrifugal experiments were performed on plasma samples obtained one week after the ligation, in order to detect any changes not observed by the simpler electrophoretic and immunochemical analyses. The patterns of LDL as well as of HDL were essentially similar to those found before operation, but the immunochemical analyses had revealed the presence of anti-HDL reacting material in the LDL-region (LDL₂). This fraction contained not only A I, but also another component in the same region in SDS gel electrophoresis as human apo E. Gel filtration of plasma in Sephadex G-200 showed that material with immunoreactivity to anti-HDL was more heterogeneously distributed and had an increased amount of larger particles after ligation of the common duct. These findings in the cholestatic LDL₂-fractions from pig are reminiscent of the observations made in HDL-region in cholestatic plasma (IV).

Cholestasis in dogs

In this study ten mongrels were used. The common bile duct was ligated in 8 dogs, while the remaining two were sham-operated. The development

of cholestasis was followed by liver function tests, which showed clear evidence of biliary obstruction. Post-mortem examination confirmed the existence of pronounced cholestasis.

In contrast with the study of biliary obstruction in pigs, agar gel electrophoresis of cholestatic canine plasma showed the presence of a cathodically migrating material, precipitable by Mn^{2+} -heparin solutions. Analogous findings in cholestatic dogs have been reported by Müller et al (54) and Ritland and Bergan (65).

Zonal ultracentrifugation of dog's plasma showed marked alterations of the LDL region after biliary obstruction for 7 days. The normal canine LDL pattern with the two partially resolved subclasses (LDL_1 and LDL_2) was changed and more complicated (illustrated in VI and VII). The LDL levels were considerably increased. This was partly due to an increase in anti- β -lipoprotein reacting material (LP B) as judged from immunochemical evidence (cf. Fig. 3 in paper VII), but distribution of LP B did not coincide with the absorbance curve at 280 nm and therefore indicated the presence of other lipoprotein species. This distribution of such lipoproteins was not explored in detail. Since the lighter part of LDL contained little LP B, this part (provisionally called CLP, paper VI) was investigated further. The small amounts of LP B were removed by hydroxyapatite chromatography, which separated the non-B part into two large fractions and one minor one. The two larger fractions showed the agar gel electrophoretic behaviour characteristic of human LP-X. The lipid composition of these fractions resembled that of human LP-X in some respects with a slight dominance of polar surface lipids, mostly phospholipids, and a very small content of triglycerides. But in one respect the canine CLP was quite different from human LP-X, namely in its cholesterol-cholesterol ester ratio, which was not reversed, but resembled that commonly found in normal plasma lipoproteins. The last-mentioned observation differs from the results of Müller et al (54), who found a pronounced dominance of unesterified cholesterol over cholesterol esters. No explanation can be offered for this discrepancy, but it is unlikely that it was caused by different preparation procedures, since preparation of the abnormal canine lipoprotein by Cohn fractionation (74) gave the same results concerning lipid composition. This was also the case for the cathodally migrating material recovered from agar gels run as described by Seidel et al (74). Studies of the lipid composition of cholestatic LDL fractions comprising the LDL class (paper VII) did not reveal any fraction with altered cholesterol-cholesterol ester ratio.

The apoprotein composition of cholestatic LDL fractions differed also from the normal canine LDL subclasses, which in addition to apo B, contained noteworthy amounts of apo A I, especially in LDL_2 , and small amounts of apoproteins with a molecular weight between 10 000 and 40 000 daltons. All these non-B apoproteins were increased in

cholestasis. Most conspicuous was the increase in the abnormal fraction isolated by hydroxyapatite chromatography, in which the components with a molecular weight of 35 - 40 000 daltons dominated the pattern.

Judging from the SDS-polyacrylamide gel patterns and the immuno-reactivity of the zonal ultracentrifugation fraction, apo A I is present in normal dog LDL (50). Apo A I is probably not present in the major B-containing LDL, but might occur in lipoproteins related to HDL, since its density distribution differs from that in man. In cholestatic dogs the HDL is decreased and its density distribution is shifted towards lower densities, which increases the amounts of apo A I in the LDL region still more. These changes were corroborated by the findings in gel filtration experiments in Sephadex G-200, where HDL related material, with a larger particle size than in normal plasma, was found in the same peak as apo B containing lipoproteins.

Although no lipoprotein with all the characteristics of human LP-X was found after ligation of the common bile duct in pigs or dogs, the changes in LDL and HDL have much in common with the changes of human lipoproteins in cholestasis. The shift in lipid composition towards a dominance of polar lipids and the appearance of apoproteins with molecular weights around 35 - 40 000 daltons in porcine and canine LDL might correspond to similar findings in human HDL, where we found an abnormal cholestatic HDL₁-C with an abundance of polar lipids and an apoprotein pattern dominated by apo E (molecular weight 35 000 daltons) (Papers III, IV). It is tempting to assume that the abnormal LDL in the animals contain particles corresponding to the human cholestatic HDL₁. This human lipoprotein most reasonably consists of the disc-like particles found in cholestatic HDL by Hamilton and Kayden (29). According to these authors, these particles are identical or very similar to "nascent" HDL, which also contains apo E (30), and human LP-X is formed from the particles by secondary events. The finding of apoproteins with molecular weights around 35 - 40 000 daltons in LDL of the cholestatic animals does not exclude the possibility of their belonging to HDL system, since even normally such material is found in the LDL class of porcine and canine plasma. It should be possible unequivocally to demonstrate the existence of non-B lipoproteins in normal and cholestatic LDL by immunosorbent techniques or other immunochemical methods. Anyhow, the present work suggests that the changes of HDL and the abnormal distribution of apoproteins probably analogous to human apo E are primary events in the formation of abnormal lipoproteins in cholestasis, and that the changes like the reversed cholesterol-cholesterol ester ratio of human lipoproteins are not of the same fundamental interest.

Diagnostic importance of lipoprotein changes in human cholestasis

The differential diagnosis of jaundice caused by obstructive and non-obstructive mechanisms is sometimes difficult despite the large number of laboratory tests available. Determination of LP-X concentrations has been suggested as a valuable tool for demonstrating cholestasis (67, 76) and for discriminating between intra- and extrahepatic obstruction (68). The purpose of the present investigation was to elucidate the changes in plasma lipoproteins, including LP-X, during cholestasis and to reconsider the value of these parameters in the differential diagnosis of jaundice.

Table II

LP-X and S-Bilirubin (S-Bil), S-alkaline phosphatase (S-ALP), S- γ -glutamyltransferase (S-GT) in 103 patients with jaundice

DIAGNOSIS	Number of patients mean (range)	Age years	S-Bil $\mu\text{mol/l}$	S-ALP $\mu\text{kat/l}$	S-GT $\mu\text{kat/l}$	LP-X Number of positive cases
CHOLELITHIASIS	42	54 (22-88)	22-425	3.5-29.3	1.4-68.1	36 (86%)
CANCER	31	68 (37-83)	45-504	5.2-63.3	1.4-55.6	31 (100%)
HEPATITIS	30	37 (16-71)	22-297	2.3-13.2	0.7-9.4	26 (87%)
REFERENCES			3-20	0.8-4.6	0.6	-

The 103 patients studied (Table II) were divided into three groups: (1) obstructive jaundice caused by gallstone, (2) obstructive jaundice due to extra- or intrahepatic malignancies and (3) jaundice due to infectious hepatitis. In patients with gallstone the diagnosis was established by X-ray examination or by surgery; in those with malignant tumour the diagnosis was confirmed by surgery and/or by histological or cytological investigations; and in those with infectious hepatitis the diagnosis was made by functional liver tests and by anamnestic data and subsequently confirmed by demonstration of Au-antigen.

α - and β -lipoprotein levels were estimated by electroimmunoassay (43). LP-X was demonstrated by crossed immunoelectrophoresis (21) against anti-C I or by electrophoresis in agar (78).

Obstructive jaundice caused by gallstones. The results of the laboratory examinations are shown in Table II. Thirty-six out of 42 patients had demonstrable LP-X. In the 6 patients with negative LP-X reaction all but one had increased activities of S-ALP and S-GT. The sixth patient with non-detectable LP-X had increased S-GT but normal S-ALP activity despite the unequivocal finding of a stone in the choledochus at surgery a few days after the examination.

The α -lipoprotein levels were normal in 33 out of the 42 cases, whereas low values were recorded in 7 patients (Fig. 2). It was noted that the patients with low α -lipoprotein levels had been jaundiced for more than 10 days. The β -lipoprotein concentration was normal in 18 out of the 42 patients, but increased in the remaining 24. As a rule, a decreased concentration of α -lipoprotein was associated with a high β -lipoprotein level.

Obstructive jaundice caused by malignant tumours. All patients had increased activities of S-ALP and S-GT, and LP-X was invariably demonstrable (Table II). The plasma α -lipoprotein concentration was decreased in 21 of the 31 cases (Fig. 2). Especially low α -lipoprotein levels were found in plasma from patients with jaundice of more than 2-3 weeks duration and in patients with S-Bil exceeding 200 mol/l. The β -lipoprotein concentration was increased in 24 patients (Fig. 3).

Infectious hepatitis. S-ALAT and S-ASAT activities were consistently increased in the 30 patients with infectious hepatitis. The S-ALP values were slightly to moderately increased in 20 of them (Table II) and S-GT activities moderately in all 30. In 26 patients LP-X was clearly demonstrable. The pattern of α - and β -lipoprotein changes was similar to that found in the patients with cancer.

Chronology of lipoprotein changes. The time-table of the changes in α - and β -lipoprotein and LP-X was studied in 15 patients with infectious hepatitis and in 7 with cancer of the pancreatic head. LP-X was detected in the first blood sample in 14 of the patients with hepatitis (Fig. 4 A). All patients with pancreatic cancer had clearly demonstrable LP-X, which disappeared about two weeks after relief of biliary obstruction by percutaneous transhepatic drainage (Fig. 4 B). In both groups β -lipoprotein and α -lipoprotein concentrations generally varied inversely with each other (Fig. 4 A and B). Despite relief of biliary obstruction the β -lipoprotein concentration continued to increase and the α -lipoprotein concentration continued to decrease another 5-6 days after which the lipoprotein levels returned to normal at the same time as LP-X disappeared.

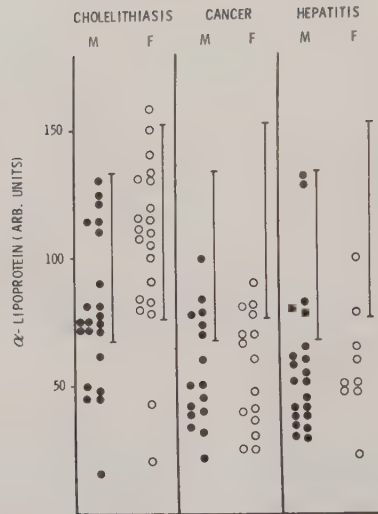


Fig. 2. Concentration of plasma α -lipoprotein in patients with jaundice caused by cholelithiasis, cancer and hepatitis. References indicated for men and women (40-60 years) were obtained by analysis of plasma samples drawn from apparently healthy individuals attending a health control

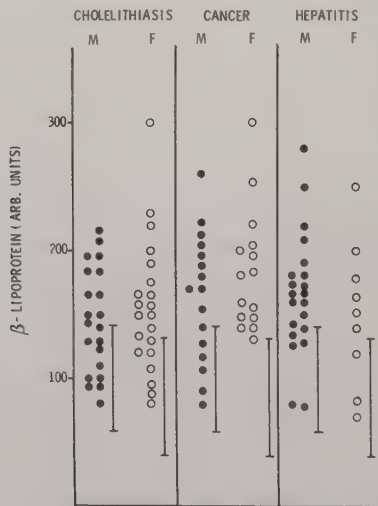


Fig. 3. Concentration of plasma β -lipoproteins in patients with jaundice caused by cholelithiasis, cancer and hepatitis. References are indicated for men and women (cf. Fig. 2).

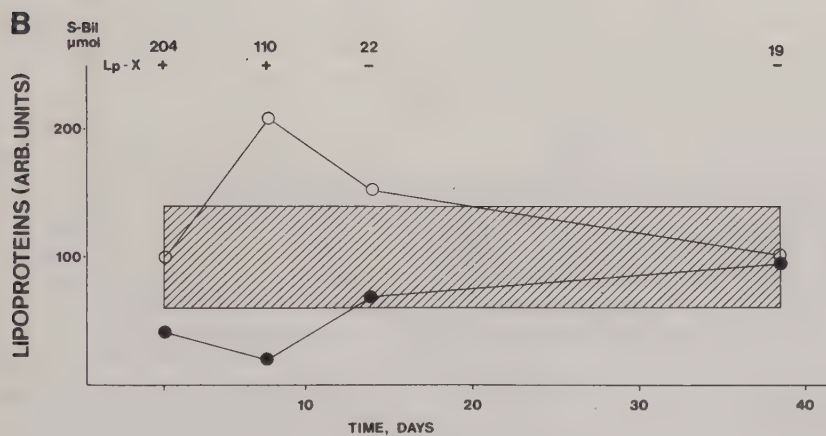
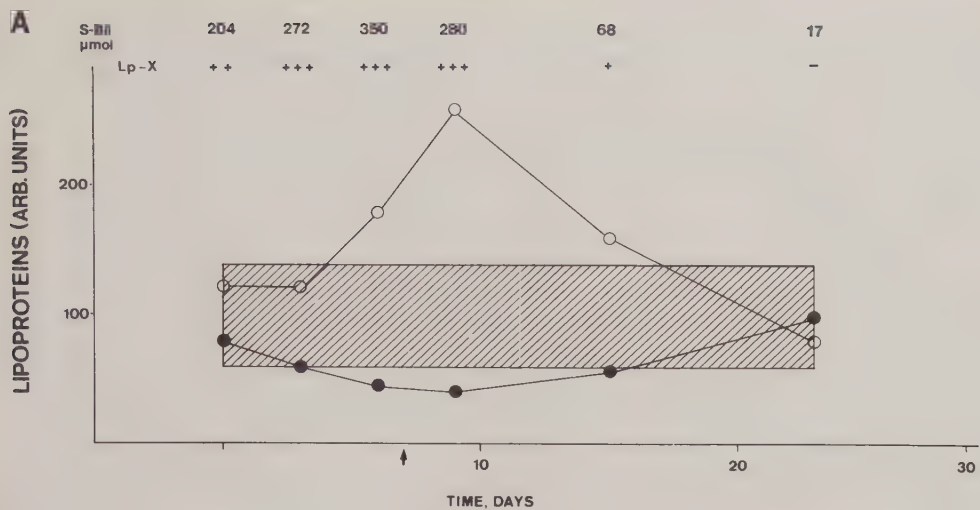


Fig. 4. The course of α - and β -lipoprotein changes in two male patients one with obstructive jaundice due to carcinoma of the pancreas (A) and the other one with jaundice caused by B-hepatitis (B).
 ●—● α -lipoprotein, ○—○ β -lipoprotein. The shaded areas represent the approximate reference intervals for α - and β -lipoproteins. A percutaneous transhepatic drainage was performed on the day marked by the arrow in (A).

Demonstration of LP-X in obstructive jaundice does not seem to be a more discriminating tool than the conventional parameters for biliary obstruction, e.g. S-ALP and S-GT activities: 6 out of 42 patients with gallstones had no detectable LP-X, whereas 26 out of 30 patients with diagnosed hepatitis had clearly demonstrable LP-X. These findings do not argue for determination of LP-X in the differential diagnosis of obstruction and hepatocellular damage. Our reluctance to include LP-X as a routine clinical test is shared by several other groups (69, 87, 91). Seidel et al and Ritland et al (67, 76) demonstrated LP-X in about 98-99% in patients with obstructive jaundice. This discrepancy between their figures and ours might perhaps be explained by differences in the selection of the patients or by technical differences between the methods used. The technique used by Seidel has given us somewhat less reproducible result in comparison with crossed immunoelectrophoresis, which also seems to be more sensitive.

Changes of α - (HDL) and β - (LDL) lipoprotein levels in plasma from patients with liver and biliary diseases have been reported (59, 77, 80). Such changes have been demonstrated with electrophoretic or ultracentrifugal separation techniques, and the increased concentrations of β - (LDL) lipoprotein have generally been ascribed to an increase in LP-X (73, 74, 84), which cannot be distinguished from (LDL) lipoprotein by these methods. The present study, in which specific immunochemical techniques were used, not only confirmed the occurrence of LP-X in biliary diseases but also demonstrated an unequivocal increase in the concentration of β -lipoprotein proper. The possibility that the observed increase of apo B-containing lipoprotein was partly due to an increase in the pre- β -lipoproteins, which also contain apo B could be excluded since crossed immunoelectrophoresis against anti-lipoprotein C, cross-reacting with VLDL, did not reveal high VLDL levels in any of the cases. Also VLDL concentrations, as estimated by zonal ultracentrifugation, were normal or decreased.

The decrease of α -lipoprotein was most obvious in hepatitis and in some of the patients with cancer (Fig. 2). In some cases with low α -lipoprotein levels the immunoprecipitates stained poorly or not at all with Sudan Black whereas the immunoprecipitates were clearly demonstrable after staining for protein. The cause of this abnormal behaviour of the immunoprecipitates is probably the decreased amount of nonpolar lipids (triglycerides and cholesterol esters) (IV).

This result with low α -lipoprotein concentrations might be a sign of the liver damage which is evident in hepatitis and possibly also present in long-standing obstructive jaundice. The underlying mechanism of these changes in lipoprotein metabolism in liver and biliary disease is obscure, but may perhaps be related to changes in the lecithin cholesterol acyltransferase (LCAT) system (24). The gel filtration pattern of plasma from one patient with LCAT deficiency

showed a particle size distribution closely resembling that seen in long-standing human cholestasis (B.G. Johansson, personal communication).

PROPOSED MECHANISMS OF THE LIPOPROTEIN ALTERATIONS IN CHOLESTASIS

The lipoprotein changes in cholestasis evidently embrace all density classes. The most well-known change is the appearance of LP-X in LDL in human cholestasis, but judging from our studies also an increase of apparently normal LDL (lipoprotein B) is demonstrable in most cases. The second event in human cholestasis is the decrease of HDL with a drastic change in the density distribution of this lipoprotein class. Finally, the VLDL is often decreased and its rate of migration abnormally low. In experimental cholestasis in pigs and dogs the lipoprotein changes observed by us differed to some extent from those found in human cholestasis; unlike published studies (54, 65) ours revealed no lipoprotein with the unique composition of human LP-X.

The mechanism(s) of these profound alterations of lipoprotein metabolism is so far poorly understood. This lack of knowledge is ascribable to no small extent to the complexity of the problem.

Seidel et al (73) suggested that LP-X was a normal short-lived lipoprotein, which was formed in the intestinal mucosa and transported in the portal circulation to the liver, where it was rapidly catabolized, and therefore undetectable in normal plasma. In cholestasis the increased concentrations of bile acids interfere with the normal catabolism of this normal lipoprotein, which in a deranged form appears in plasma LDL class.

This hypothesis was recently abandoned by Seidel's group (52) who reported evidence in the support of a new hypothesis, suggesting that LP-X is derived from a "precursor" lipoprotein complex, which is normally excreted by the liver into bile, but which refluxes in obstructive jaundice into plasma, where it appears as LP-X. Their view was based on the following observations: (1) bile lipoproteins can be converted to LP-X in vitro by addition of albumin or serum to native bile; (2) bile lipoprotein has a lipid composition identical with LP-X; (3) LP-X can be converted to bile-LP-like particles by addition of bile salts to a LP-X positive serum; (4) LP-X-like material appears in plasma a few hours after establishment of an experimental open communication between the common bile duct and vena cava in dogs.

This hypothesis does not explain the appearance of LP-X in plasma in patients with familial LCAT deficiency who consistently exhibit LP-X (85) despite normal liver function and no signs of biliary obstruction.

It should be noted that electron microscopic studies of the liver in obstructive jaundice have not shown the presence of LP-X or similar particles within the liver (11, 82).

The above-mentioned hypothesis concerning formation of LP-X does not take into account the fact that also HDL is profoundly altered in cholestasis. This might be an independent phenomenon, but though the changes of HDL is a comparatively late event, there might, perhaps, be some connection between the appearance of LP-X and the alteration of the HDL. Hamilton and Kayden (29) postulated the following mechanism for the lipoprotein changes. HDL is synthesized in the liver and secreted as discoid, "nascent" HDL particles, which are rapidly converted to normal HDL in plasma by the action of LCAT. This conversion might be inhibited in cholestasis either by a decrease of the LCAT concentration or possibly by an impairment of LCAT activity by bile salts, acting by displacement of the LCAT cofactor apo A I from the HDL particle. The substitution of the structural component apo A I by bile salts would also give rise to a less stable "nascent" HDL particle, which may be easily transformed to a collapsed vesicle (LP-X). Theoretically, this hypothetical model would explain why cholestatic plasma contains "nascent" HDL and LP-X as well as normal serum HDL.

The lipoprotein patterns we have found in human obstructive jaundice correlate well with Hamilton's and Kayden's hypothesis, and are consistent with a relative LCAT-deficiency. The changes observed also show similarities with the more pronounced alterations seen in familial LCAT deficiency (24, 58). Thus, judging from its composition (predominance of polar lipids and apo E) the new component found in cholestatic plasma (HDL₁-C) closely resembles "nascent" HDL (29) as well as the discoidal HDL found in LCAT deficiency, and LP-X is demonstrable in both conditions.

So far, there is no conclusive evidence that LP-X is formed by abnormal conversion of nascent HDL particles. The late appearance of HDL changes might possibly argue against such a mechanism.

The lipoprotein changes in experimental cholestasis (I, II and III) were reminiscent of the HDL changes in human cholestatic plasma. Although no drastic decrease in HDL was noted in dogs or pigs after biliary obstruction, there was some evidence of a changed distribution of lipoprotein particles related to HDL. Furthermore, the change of lipid composition and the appearance in LDL of new apoproteins possibly analogous to human apo E suggested that these particles might derive from nascent HDL. Further studies of the abnormal lipoprotein particles, including electron microscopy and identification of the apoproteins, are needed before any conclusion can be drawn about their relation to human cholestatic HDL₁.

No explanation can be offered for the mechanisms responsible for the apparent increase of "normal" LDL (LP B) which is reported in the present work. But in the light of the general consensus of opinion

about the fate of normal LDL (27) one might imagine that the elimination of these lipoprotein particles is impaired by some defect in the function of cell receptors essential for the uptake of LDL before catabolism, or that the increase of this lipoprotein fraction might be due to an increased production of LDL from VLDL and IDL, a possibility consistent with the low VLDL levels often observed. Without further relevant knowledge, any explanation of the cause of the increase in the LDL cannot be more than speculative.

SUMMARY

The present work comprises studies of plasma lipoproteins in human cholestasis and in experimental biliary obstruction induced in dogs and pigs. Lipoproteins belonging to the VLDL-LDL class and HDL class were separated by zonal ultracentrifugation and characterized immunochemically and by studies of their apoprotein and lipid composition.

Zonal ultracentrifugation revealed a variety of VLDL-LDL patterns in human cholestasis. In half of the cases a simple pattern was revealed with a distinct LP-X fraction well-separated from LP B. In the remaining cases more complicated patterns were found, necessitating further fractionation steps for the isolation of LP-X. The VLDL peak was most often low. Immunochemical studies of the β -lipoprotein-pre- β -lipoprotein patterns with crossed immunoelectrophoresis against anti- β -lipoprotein or anti-apo C revealed distinct changes. Crossed immunoelectrophoresis and electroimmunoassay with an intermediate antibody gel was proposed for specific analysis of LP-X.

Changes in the HDL class in patients with obstructive jaundice were most simply revealed by a marked decrease of the α -lipoprotein zone in agarose gel electrophoresis. HDL changes occurred in long-standing biliary obstruction. Crossed immunoelectrophoresis demonstrated an altered pattern against anti-HDL showing a decrease of the peak with α -mobility and the appearance of several additional peaks. Gel filtration and zonal ultracentrifugation also showed a changed distribution of HDL. On zonal ultracentrifugation a new HDL subclass (HDL₁-C) appeared with a peculiar protein and lipid composition: predominance of apo E and an inverse cholesterol-cholesterol ester ratio. HDL₂ and HDL₃ were reduced and the distinct peaks normally formed by these subclasses were missing. The changes demonstrated are similar to those found in familial LCAT-deficiency.

Experimental cholestasis was induced in pigs and dogs. Changes in LDL and HDL patterns occurred in both species but no protein with the characteristic composition of human LP-X could be detected. In the dogs the appearance of LDL-components not containing apo B were demonstrated by zonal ultracentrifugation and hydroxyapatite chromatography. Studies of the lipid composition of these components did not reveal the inversed ratio of cholesterol-cholesterol ester characteristic of human LP-X. The protein part was dominated by A I and two components with molecular weights 35 - 40 000 daltons. Also other procedures used in the preparation of cholestatic canine lipoproteins (Cohn fractionation, preparative agar gel electrophoresis) failed to produce any lipoprotein with an inverse cholesterol-cholesterol ester ratio.

A clinical study of lipoprotein was performed in patients with hepatitis or biliary obstruction due to gallstones or cancer. LP-X was detected in 86% of the patients with gallstones or hepatitis and in all cases with cancer. Increased levels of β -lipoprotein was also a common finding. Low α -lipoprotein levels were found especially in cases of hepatitis and in cholestasis with a duration of more than two weeks. Possible mechanisms for the lipoprotein alterations in clinical and experimentally induced biliary obstruction are discussed.

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REFERENCES

1. Abell, L.L., Levy, B.B., Brodie, B.B. & Kendall, F.A.: A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* 195, 357, 1952.
2. Alaupovic, P., Lee, D.M. & Mc Conathy, W.J.: Studies on the composition and structure of plasma lipoproteins. Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. *Biochim. Biophys. Acta* 260, 689, 1972.
3. Anderson, N.G. & Burger, C.L.: Separation of cell components in the zonal ultracentrifuge. *Science* 136, 646, 1962.
4. Axelsen, N.H.: Intermediate gel in crossed and in fused rocket immunoelectrophoresis. *Scand. J. Immunol.* 2, Suppl. 1, 71, 1973.
5. Bartlett, G.R.: Phosphorous assay in column chromatography. *J. Biol. Chem.* 234, 466, 1959.
6. Belfrage, P., Wiebe, T. & Lundquist, A.: Methods for the determination in the nanomole range of lipids in liver fine-needle aspiration biopsies. *Scand. J. Clin. Lab. Invest.* 26, 53, 1970.
7. Blomhoff, J.B.: High density lipoproteins in cholestasis. *Scand. J. Gastroent.* 9, 591, 1974.
8. Burstein, M., Scholnik, H.R. & Morfin, R.: Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid. Res.* 11, 583, 1970.
9. Calandra, S., Montaguti, M., Cartoni, V. & Pasquali-Ronchetti, I.: Plasma lipoproteins in rats with experimental biliary obstruction. I. A chemical study. *Biochim. Biophys. Acta* 409, 1, 1975.
10. Chanutin, A. & Ludewig, S.: The blood plasma cholesterol and phospholipid phosphorus in rats following partial hepatectomy and following ligation of the bile duct. *J. Biol. Chem.* 115, 1, 1936.
11. Cooper, A.D., Jones, A.L., Koldinger, R.E. & Ockner, R.K.: Selective biliary obstruction: a model for the study of lipid metabolism in cholestasis. *Gastroenterology* 66, 574, 1974.
12. Davis, B.J.: Disc electrophoresis - II Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* 121, 404, 1964.

13. Eder, H.A., Russ, E.M., Rees Pritchett, R.A., Wilber, M.M. & Barr, D.P.: Protein-lipid relationships in human plasma: in biliary cirrhosis, obstructive jaundice and acute hepatitis. *J. Clin. Invest.* 34, 1147, 1955.
14. Ekman, R., Johansson, B.G. & Ravnskov, U.: Gel chromatography on Sephadex gels with narrow particle size distribution obtained by dry elutriation. *Anal. Biochem.* 70, 628, 1976.
15. Ekman, R. & Nilsson-Ehle, P.: Effects of apolipoproteins on lipoprotein lipase activity of human adipose tissue. *Clin. Chim. Acta.* 63, 29, 1975.
16. Epstein, E.Z.: Cholesterol of the blood in hepatic and biliary diseases. *Arch. intern. Med.* 50, 203, 1932.
17. Felts, J.M., Itakura, H. & Crane, R.T.: The mechanism of assimilation of constituents of chylomicrons, very low density lipoproteins and remnants - a new theory. *Biochim. Biophys. Res. Commun.* 66, 1467, 1975.
18. Flint, A., Jr.: Experimental researches into a new excretory function of the liver: consisting in the removal of cholestasis from the blood, and its discharge from the body in the form of stercorine. *Amer. J. Med. Sci.* 44, 305, 1862.
19. Folch, J., Lees, M. & Sloane Stanley, G.H.: A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497, 1957.
20. Fredrickson, D.S. & Levy, R.I.: Familial hyperlipoproteinemia, the metabolic basis of inherited disease. Third edition. (ed) S.B. Stanbury, J.B. Wyngaarden, D.S., Fredrickson. New York Mc Graw - Hill Book Company. 545, 1972.
21. Ganrot, P.O.: Crossed immunoelectrophoresis. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124, 39, 1972.
22. Garrard, W.T. & Bonner, J.: Changes in chromatin proteins during liver regeneration. *J. Biol. Chem.* 249, 5570, 1974.
23. Glomset, J.A.: The plasma lecithin cholesterol acyltransferase reaction. *J. Lipid. Res.* 9, 155, 1968.
24. Glomset, J.A., Norum, K.R., Nichols, A.V., Forte, T., King, W.C., Albers, J., Mitchell, C.D., Applegate, K.R. & Gjone, E.: Plasma lipoprotein metabolism in familial lecithin: cholesterol acyltransferase deficiency. *Scand. J. Clin. Lab. Invest.* 33, Suppl. 137, 165, 1974.

25. Glueck, C.J., Fallat, R.W., Millett, F., Gartside, P., Elston, R.C. & Go, R.C.P.: Familial hyper- α -lipoproteinemia: studies in eighteen kinders. *Metabolism*. 24, 1243, 1975.
26. Gofman, J.W., Delalla, O., Glazier, F., Freeman, N.K., Lindgren, F.T., Nichols, A.V., Strisower, B. & Tamplin, A.R.: The serum lipoprotein transport system in health metabolic disorders, atherosclerosis and coronary heart disease. *Plasma* 2, 413, 1954.
27. Goldstein, J.C. & Brown, M.S.: Familial hypercholesterolemia: a genetic regulatory defect in cholesterol metabolism. *Am. J. Med.* 58, 147, 1975.
28. Hamilton, R.L., Havel, R.J., Kane, J.P., Blaurock, A.E. & Sata, T.: Cholestasis: Lamellar structure of the abnormal human serum lipoprotein. *Science* 172, 475, 1971.
29. Hamilton, R.L. & Kayden, H.J.: The liver and the formation of normal and abnormal plasma lipoproteins. In *The Liver: Normal and Abnormal Functions*. F.F. Becker, (ed), Marcel Dekker, Inc., New York. Part A: 531, 1974.
30. Hamilton, R.L., Williams, M.C., Fielding, J. & Havel, R.J.: Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* 58, 667, 1976.
31. Havel, R.J., Eder, H.A. & Bragdon, J.H.: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34, 1345, 1955.
32. Itaya, K. & Ui, M.: A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta.* 14, 361, 1966.
33. Johansson, B.G.: Agarose gel electrophoresis. *Scand. J. Clin. Lab. Invest.* 29. Suppl. 124, 7, 1972.
34. Jones, J.W. & Ways, P.: Abnormalities of high density lipoproteins in a beta-lipoproteinemia. *J. Clin. Invest.* 46, 1151, 1967.
35. King, J. & Laemmli, U.K.: Polypeptides of the tail fibres of bacteriophage T 4. *J. Mol. Biol.* 62, 465, 1971.
36. Komiang, P., Bensadoun, A. & Wang Yang, M.W.: Effect of an anti-lipoprotein lipase serum on plasma triglyceride removal. *J. Lipid Res.* 17, 498, 1976.
37. Kostner, G. & Alaupovic, P.: Studies of the composition and structure of plasma lipoproteins. Separation and quantification of the lipoprotein families occurring in the high density lipoproteins of human plasma. *Biochemistry* 11, 3419, 1972.

38. Kostner, G.M., Patsch, J.R., Sailer, S., Braunsteiner, H. & Holasek, A.: Polypeptide distribution of the main lipoprotein density classes separated from human plasma by rate zonal ultracentrifugation. *Eur. J. Biochem.* 45, 611, 1974.
39. Kostner, G.M., Pelek, W. & Holasek, A.: Immunochemical measurement of lipoprotein-X. *Clin. Chem.* 20, 676, 1974.
40. Kostner, G.M., Laggner, P., Prexl, H.J., Holasek, A., Ingolic, E. & Geymayer, W.: Investigation of the abnormal low-density lipoprotein occurring in patients with obstructive jaundice. *Biochem. J.* 157, 401, 1976.
41. Kruski, A.W. & Narwayan, K.A.: A simplified procedure for protein determination of turbid lipoprotein samples. *Anal. Biochem.* 47, 299, 1972.
42. Kunkel, H.G. & Ahrens, E.H. Jr.: The relationship between serum lipids and the electrophoretic pattern with particular reference to patients with primary biliary cirrhosis. *J. Clin. Invest.* 28, 1575, 1949.
43. Laurell, C.B.: Electroimmuno assay. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124, 21, 1972.
44. Laurell, S. & Tibbling, G.: An enzymatic fluorometric micromethod for the determination of glycerol. *Clin. Chim. Acta* 13, 317, 1966.
45. Levy, R.I.: The plasma lipoproteins: An overview. In Jamiesson, G.A. and Greenwalt, T.J. (ed): *Progress in clinical and biological research* vol. 5, 25, 1976.
46. Lindgren, F.T., Jensen, L.C. & Hatch, F.T.: The isolation and quantitative analysis of serum lipoproteins. In Nelson, G.J. (ed): *Blood lipids and lipoproteins*. New York, Interscience 181, 1972.
47. McConathy, W.J. & Alaupovic, P.: Isolation and partial characterization of apolipoprotein D: A new protein moiety of human plasma lipoprotein system. *FEBS Letters* 37, 178, 1973.
48. Magnani, H.N. & Alaupovic, P.: A method for the quantitative determination of the abnormal lipoprotein (LP-X) of obstructive jaundice. *Clin. Chim. Acta.* 38, 405, 1972.
49. Man, E.B., Kartin, B.L., Durlacher, S.H. & Peters, J.P.: The lipids of the serum and liver in patients with hepatic diseases. *J. Clin. Invest.* 24, 623, 1945.

50. Mahley, R.W. & Weisgraber, K.H.: Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoproteins from control dogs. *Circulation Research* 35, 713, 1974.
51. Mancini, G., Carbonara, A. & Heremans, J.F.: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem.* 2, 235, 1965.
52. Manzato, E., Fellin, R., Baggio, G., Walch, S., Neubeck, W. & Seidel, D.: Formation of lipoprotein-X. *J. Clin. Invest.* 57, 1248, 1976.
53. Morrisett, J.D., Jackson, R.L. & Gotto, A.M.: Lipoproteins: structure and function. In *Annual Review of Biochemistry* vol 44, 183, 1975.
54. Müller, P., Fauser, U., Fellin, R., Wieland, H. & Seidel, D.: Isolation and characterization of lipoprotein-X (Lp-X) from canine. *FEBS Letters* 38, 53, 1973.
55. Nelson, G.J.: Blood lipids and lipoproteins: Quantitation, Composition and Metabolism. New York, Interscience 1972.
56. Neubeck, W. & Seidel, D.: Direct method for measuring lipoprotein-X' in serum. *Clin. Chem.* 21, 853, 1975.
57. Noble, R.P.: Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* 9, 693, 1968.
58. Norum, K.R., Glomset, J.A., Nichols, A.V., Forte, T., Albers, J.J., King, W.C., Mitchell, C.D., Applegate, K.R., Gong, E.L., Cabana, V. & Gjone, E.: Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: effects of incubation with lecithin: cholesterol acyltransferase in vitro. *Scand. J. clin. Lab. Invest.* 35, Suppl. 142, 31, 1975.
59. Papadopolus, N.M. & Charles, M.N.: Serum lipoprotein patterns in liver disease. *Proc. Soc. Exp. Biol. (N.Y.)*, 134, 797, 1970.
60. Patsch, J.R., Patsch, W., Sailer, S. & Braunsteiner, H.: Isolation and partial characterization of two abnormal human plasma lipoproteins: Lp-X₁ and Lp-X₂. *Biochim. et Biophys. Acta* 434, 419, 1976.
61. Patsch, J.R., Sailer, S. & Braunsteiner, H.: Lipoprotein of the density 1.006 - 1.020 in plasma of patients with type III hyperlipoproteinemia in the postabsorptive state. *Europ. J. Clin. Invest.* 5, 45, 1975.

62. Quarfordt, S.H., Oelschlaeger, H. & Krigbaum, R.W.: Liquid crystalline lipid in the plasma of humans with biliary obstruction. *J. Clin. Invest.* 51, 1979, 1972.
63. Quarfordt, S.H., Oelschlaeger, H., Krigbaum, W.R., Jakobi, L. & Davis, R.: Effect of biliary obstruction on canine plasma and biliary lipids. *Lipids* 8, 522, 1973.
64. Redgrave, T.G.: Formation of cholesterol ester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* 49, 465, 1970.
65. Ritland, S. & Bergan, A.: Plasma concentration of lipoprotein-X (Lp-X) in experimental bile duct obstruction. *Scand. J. Gastroent.* 10, 17, 1975.
66. Ritland, S.: A method for quantitative determination of the abnormal lipoprotein of cholestasis, LP-X. *Clin. Chim. Acta.* 55, 359, 1974.
67. Ritland, S., Blomhoff, J.P., Elgjo, K. & Gjone, E.: Lipoprotein-X (LP-X) in liver disease. *Scand. J. Gastroent.* 8, 155, 1973.
68. Ritland, S.: Quantitative determination of the abnormal lipoprotein of cholestasis, LP-X, in liver disease. *Scand. J. Gastroent.* 10, 5, 1975.
69. Ross, A., Murphy, G.M., Wilkinson, P.A., Mills, G.L. & Sherlock, S.: Occurrence of an abnormal lipoprotein in patients with liver disease. *Gut* 11, 1035, 1970.
70. Röschlau, P., Bernt, E. & Gruber, W.: Enzymatische Bestimmung des Gesamt Cholesterins im Serum. *Z. Klin. Chem. Klin. Biochem.* 12, 403, 1974.
71. Scanu, A. & Hughes, W.L.: Further characterization of the human serum D. 1.063 - 1.21, α -lipoprotein. *J. Clin. Invest.* 41, 1681, 1962.
72. Scanu, A., Toth, J., Edelstein, C., Koga, S. & Stiller, E.: Fractionation of human serum high density lipoprotein in urea solutions. Evidence for polypeptides heterogeneity. *Biochemistry* 8, 3309, 1969.
73. Seidel, D., Alaupovic, P. & Furman, R.H.: A lipoprotein characterizing obstructive jaundice, I. Method for quantitative separation and identification of lipoproteins in jaundiced subjects. *J. Clin. Invest.* 48, 1211, 1969.

74. Seidel, D., Alaupovic, P., Furman, R.H. & Mc Conathy, W.J.: A lipoprotein characterizing obstructive jaundice. II. Isolation and partial characterization of the protein moieties of low density lipoproteins. *J. Clin. Invest.* 49, 2396, 1970.
75. Seidel, D., Büff, H.U., Fauser, U. & Bleyl, U.: On the metabolism of lipoprotein-X (LP-X). *Clin. Chim. Acta.* 66, 195, 1976.
76. Seidel, D., Gretz, H. & Ruppert, C.: Significance of the LP-X test in differential diagnosis of jaundice. *Clin. Chem.* 19, 86, 1973.
77. Seidel, D., Greten, H., Geisen, H.P., Wengeler, H. & Wieland, H.: Further aspects on the characterization of high and very low density lipoproteins in patients with liver disease. *Europ. J. Clin. Invest.* 2, 359, 1972.
78. Seidel, D., Wieland, H. & Ruppert, C.: Improved techniques for assessment of plasma lipoprotein patterns. I. Precipitation in gels after electrophoresis with polyanionic compounds. *Clin. Chem.* 19, 737, 1973.
79. Shore, V.G. & Shore, B.: Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry* 12, 502, 1973.
80. Smith, S.C., Scheig, R.L., Klatskin, G. & Levy, R.I.: Lipoprotein abnormalities in liver disease. *Clin. Res.* 15, 330, 1967.
81. Sniderman, A.D., Carew, R.E., Chandler, J.G. & Steinberg, D.: Paradoxical increase in rate of catabolism of low density lipoproteins after hepatectomy. *Science* 183, 526, 1974.
82. Stein, O., Alkan, M. & Stein, Y.: Obstructive jaundice lipoprotein particles studied in ultrathin sections of livers of bile duct-ligated mice. *Lab. Invest.* 29, 166, 1973.
83. Steinbuch, M. & Audran, R.: Preparation of human immunoglobulin by caprylic acid precipitation. *Arch. Biochem.* 134, 279, 1969.
84. Switzer, S.: Plasma lipoproteins in liver disease: I. Immunologically distinct low density lipoproteins in patients with biliary obstruction. *J. Clin. Invest.* 46, 1855, 1967.
85. Torsvik, H., Berg, K., Magnani, H.N., McConathy, W.J., Alaupovic, P. & Gjone, E.: Identification of the abnormal cholestatic lipoprotein (LP-X) in familial LCAT-deficiency. *FEBS Letters* 24, 165, 1972.

86. Utermann, G., Menzel, H.J. & Langer, K.H.: On the polypeptide composition of an abnormal high density lipoprotein (LP-E) occurring in LCAT-deficient plasma. FEBS Letters 45, 29, 1974.
87. Vergani, C., Pietrogrande, M. & Crondona, M.C.: Study of the abnormal lipoprotein-X in obstructive and non-obstructive jaundice. Clin. Chim. Acta. 48, 243, 1973.
88. Weber, K. & Osborn, M.: The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406, 1969.
89. Wengeler, H., Greten, H. & Seidel, D.: Serum cholesterol esterification in liver disease. Combined determinations of lecithin: cholesterol acyltransferase and lipoprotein X. Europ. J. Clin. Invest. 2, 372, 1972.
90. Wilcox, H.J., Davies, D.C. & Heimberg, M.: The isolation of lipoproteins from human plasma by ultracentrifugation in zonal rotors. J. Lipid Res. 12, 160, 1971.
91. Witt, I. & Ober, M.: Lipoprotein-X bei Neugeborenen: Gehäuftes Auftreten ohne nachweisbare Cholestase. J. Clin. Chem. Clin. Biochem. 14, 197, 1976*

